

AD _____

Award Number: DAMD17-03-1-0745

TITLE: Neurotrophin Therapy of Neurodegenerative Disorders with Mitochondrial Dysfunction

PRINCIPAL INVESTIGATOR: Linda L. Bambrick, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland, Baltimore
Baltimore, MD 21201

REPORT DATE: September 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-09-2006		2. REPORT TYPE Annual		3. DATES COVERED 1 Sep 2005 – 31 Aug 2006	
4. TITLE AND SUBTITLE Neurotrophin Therapy of Neurodegenerative Disorders with Mitochondrial Dysfunction				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-03-1-0745	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Linda L. Bambrick, Ph.D. E-Mail: Lbambric@umaryland.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Maryland, Baltimore Baltimore, MD 21201				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT This research program will determine whether accelerated neuron death due to increased oxidative stress resulting from mitochondrial dysfunction can be compensated or corrected by neurotrophin stimulation. The experiments will be carried out in two models of mitochondrial dysfunction. 1)hippocampal neurons from the trisomy 16 mouse, which undergo increased apoptosis and have a mitochondrial defect, that has now been identified as a decrease in Complex I-mediated respiration and altered mitochondrial protein expression and 2)neurons chronically treated with the neurotoxin rotenone to induce a defect in mitochondrial function. 0.1-0.5 nM rotenone treatment has now been shown to leave hippocampal neurons vulnerable to a second oxidative stress. A unique aspect of this approach is that the neuronal responsiveness to brain derived neurotrophic factor (BDNF) will be enhanced by by breeding to a mouse line with altered BDNF receptor expression. Neurons with an enhanced response to endogenous BDNF may be more resistant to oxidative stress characteristic of Parkinson's disease and other neurodegenerative disorders.					
15. SUBJECT TERMS mitochondria, neurotrophin, BDNF, trkB, trisomy 16, oxidative stress, rotenone, Parkinsons					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Cover letter.....	page 1
Form SF298.....	page 2
Table of Contents.....	page 3
Introduction.....	page 4
Body of report.....	page 4
Key accomplishments.....	page 9
Conclusions.....	page 9
References.....	page 10

Introduction

This research program will determine whether accelerated neuron death due to mitochondrial dysfunction can be compensated for or corrected by neurotrophin stimulation. This study started in the trisomy 16 mouse (Ts16) where neurons undergo increased apoptosis (Bambrick and Krueger, 1999). In this mouse we had previously shown a defect in neurotrophin signaling through the trkB receptor/brain-derived neurotrophic factor (BDNF) ligand signaling pathway (Dorsey et al., 2002), while we and other researchers had found increased oxidative stress (Schuchmann and Heinemann, 2000). I proposed that there was a mitochondrial defect in Ts16 that acted together with the trkB signaling defect to cause neuron death. As a part of the present research program, I have previously demonstrated (Bambrick et al., 2005; Progress Report 2005) that there is a defect in mitochondrial respiration that is specific for Complex I of the electron transport chain, the same complex that is implicated in Parkinson's disease (PD). (Figure 1).

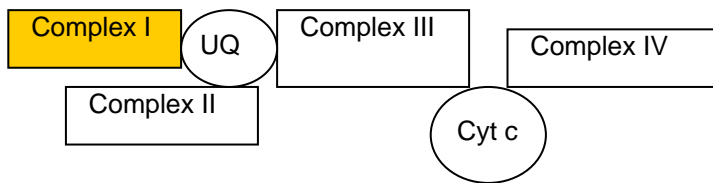


Figure 1A. Diagrammatic representation of the Electron Transport Chain (ETC). Fatty acid oxidation and the TCA cycle generate NADH or FADH₂ from substrates such as glutamate/malate or succinate, respectively. Complex I transfers electrons from NADH to ubiquinone (UQ), while Complex II does the same with electrons from FADH₂. Complex III takes electrons from UQ and transfers them to cytochrome c (cyt c). Electrons from cyt c are transferred to O₂ by Complex IV to give H₂O. During this process, protons are pumped out of the mitochondria to generate the electrochemical gradient that is used by the ATP synthetase to make ATP.

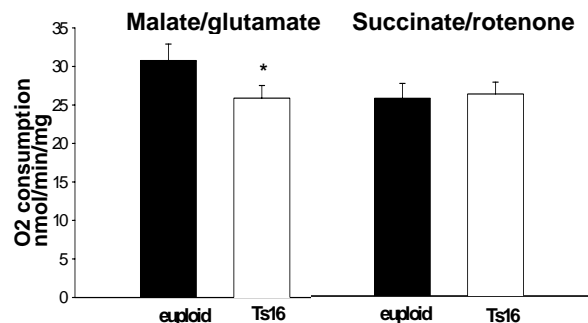


Figure 1B: Selective impairment of Complex I-mediated State 3 respiration in Ts16. Oxygen consumption was measured in crude mitochondrial fractions from embryonic day 16 brain using the minichamber and a Clarke-type electrode from Strathkelvin. Data are means and SEM for 4-6 preparations. * significantly different from euploid, $p < 0.05$.

In the past year I have now established that there is also a pattern of decreased expression of mitochondrial proteins in Ts16 that is most consistent with an increased oxidative stress (Figures 2-5). Experiments are now underway using Ts16xTK1^{-/-} mice (Dorsey et al., 2006) to test whether the effects of this increased oxidative stress are reversed when BDNF signaling is restored. In studies of living cells using hippocampal neurons from the Ts16 and control mice I have now shown that there is less mitochondrial NAD(P)H in Ts16 (Figure 6). This is also consistent with increased oxidative stress and has opened up a new therapeutic possibility since other work in our research group has shown that PARP inhibitors can protect NAD(P)H levels under oxidative stress in vitro. Finally, given that Ts16 cells have altered intracellular calcium and altered trkB expression, I have been involved in a collaboration on calcium regulation of BDNF and trkB (Kingsbury et al., 2003, 2006). I have used techniques from this collaboration to look at BDNF regulation in astrocytes as part of a novel hypothesis for the role of astrocytes in Parkinson's disease. Preliminary data has shown the feasibility of this approach (Figure 7) and this is now going to be developed for a separate research grant.

Research Accomplishments Year Three:

Electron Chain Transport (ETC) proteins in Ts16.

The members of the ETC (Figure 1A and Complex V, the ATP synthase) are multiprotein complexes. Mammalian Complex I, for example, is composed of 45-46 different subunits--7 encoded by mitochondrial and the rest by nuclear DNA. To study their regulation, Mitosciences (Eugene OR) has developed antibodies

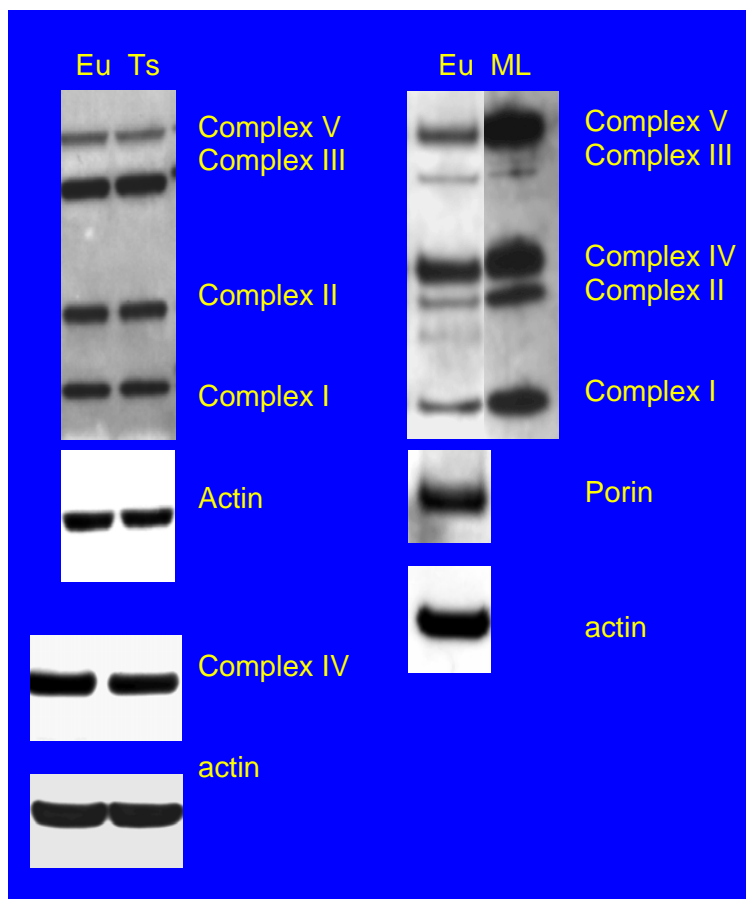


Figure 2: Western blots of mouse cortex homogenates using the ETC detection cocktail MS601, and an anti-COXIV, subunit I antibody in euploid and Ts16 cortex (left) and MS604 and porin in euploid cortex and rat liver, right. Expression of actin is shown in both panels.

against those subunits of the complexes that are labile when their complex is not assembled and thus most likely to reflect changes in protein expression or damage. After I found a selective defect in Complex I-mediated respiration (Figure 1B), I asked whether there was a selective loss of Complex I in Ts16 mitochondria. To address this question, Western blot analysis was performed on the fractions used for respiration experiments with an anti-human ETC cocktail (Mitosciences, MS-601), normalizing the results to actin. These experiments (Progress Report 2005) found no significant change in Ts16, relative to euploid, but very large variability in the results for both Ts16 and euploid cortical mitochondria. This was probably due to differing amounts of actin contaminating the mitochondrial fraction pellet, which may also have prevented the detection of changes in Ts16 brain. I therefore repeated these experiments using homogenates of euploid and Ts16 brain normalizing to both the mitochondrial outer membrane protein porin and to actin. I used homogenates so that any differences in Ts16 mitochondrial properties or amounts would not affect the outcome and normalized to porin to measure amounts per

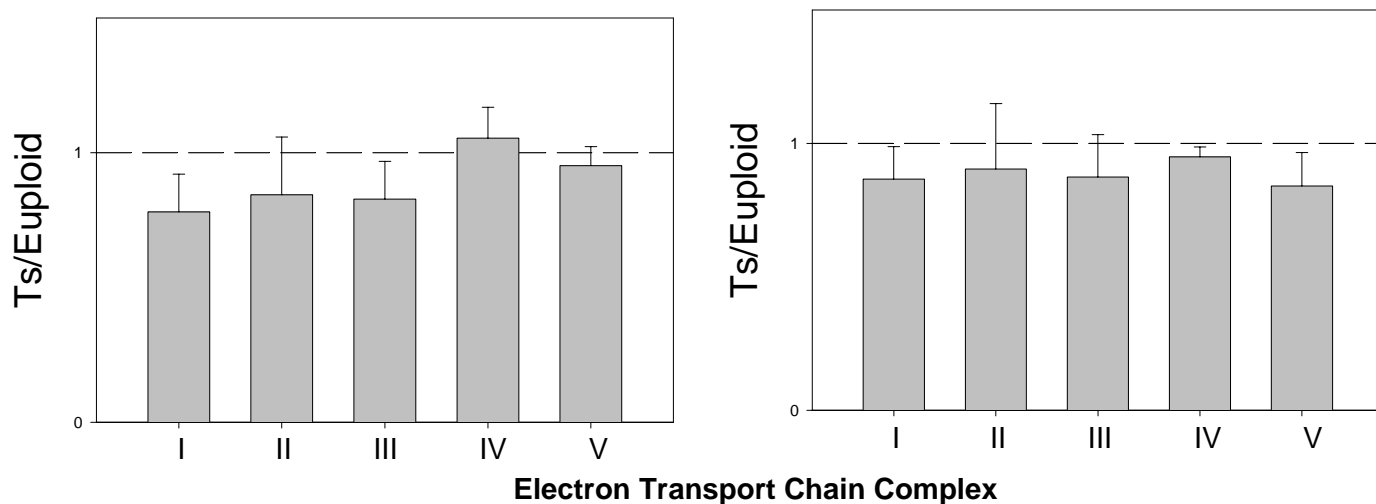


Figure 3: ETC protein expression in euploid and Ts16 hippocampus (left) and cortex (right). Means and SD for 5-7 experiments using MS601 and anti-COXIV subunit I normalized to porin expression.

mitochondria and to actin to control for overall changes in the number of mitochondria/cell. Homogenates were prepared from hippocampus and from cortex, separately, since hippocampus is more sensitive to damage in ischemia and our neuron preparations are from hippocampus. Homogenates were isolated as follows: E17 mouse cortices were homogenized on ice in 225 mM mannitol, 75 mM sucrose, 5 mM HEPES pH 7.4 with 0.5

mM EDTA. Homogenates were centrifuged at 1,250 X g X 4 min, the pellet discarded and the supernatant either used immediately or frozen at -20°C . Homogenates were prepared from three-five pooled euploid (control) and Ts16 littermates.

Figure 2 shows Western blots for euploid and Ts16 cortex using the anti-human ETC protein cocktail (MS601, Mitosciences). The antihuman Complex IV in this cocktail does not recognize rodent Complex IV, so this was detected separately using an antibody to the Complex IV protein COX IV, subunit I (Invitrogen, CA). Data were normalized to porin or to actin and then Ts16 levels were expressed relative to the euploid bands from the same prep and day. There was no difference in the porin/actin ratio (Table 1), suggesting that there is no difference in the mitochondrial mass/unit cellular protein in Ts16—although, interestingly, there was a decrease in the amount of mitochondrial protein in hippocampus as compared to cortex for both mice.

	Cortex	Hippocampus
Euploid	1.0	0.82 \pm 0.09
Ts16	0.99 \pm 0.03	0.80 \pm 0.08

Table 1: Porin/actin ratio normalized to euploid cortex. Data are from experiments where cortex and hippocampal samples from the same brain and euploid and Ts brains from the same litter were run on a single western blot Mean \pm SEM, n= 6

The data are presented normalized to porin with Ts16 expressed as a fraction of the euploid ETC/porin (Figure 3). More recently, Mitosciences has developed an anti-rodent ETC cocktail (MS604, Figure 2). The data obtained using this cocktail and probing a new set of euploid and Ts16 cortex and hippocampus homogenates are shown in Figure 4. Here the percent change from euploid is shown in a box whisker plot, which gives a better idea of the distribution of the data than a mean and SD. There is a consistent pattern of decreased ETC protein expression in Ts16 for complexes I-III and V, but not IV using either antibody cocktail (Figures 3,4) . The median change is negative in 9 of 10 bands, a finding that would occur only about 2% of the time if euploid and Ts16 protein levels were not different and any observed difference occurred by random chance (from the binomial distribution). Direct comparison of the raw band intensity normalized to porin for Complex I from euploid and Ts16 brain found a significant reduction by paired t-test ($p<0.05$).

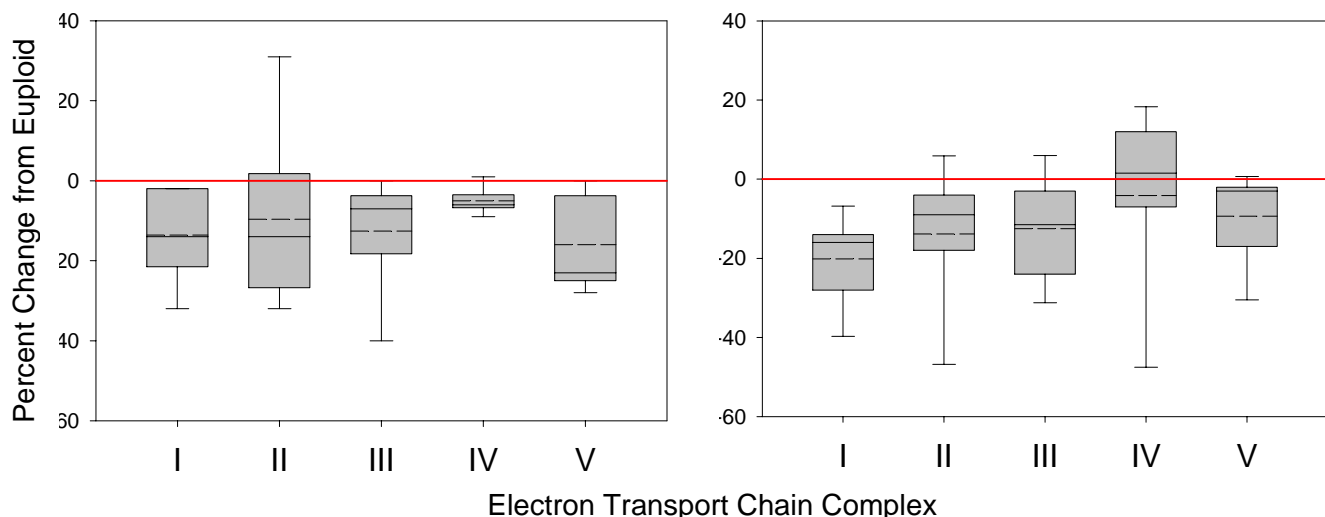


Figure 4: Electron transport chain proteins detected with an anti-rodent monoclonal antibody cocktail (MS604, Mitosciences, Figure 2B) in hippocampal (left) and cortical (right) homogenates from Ts16 brains. Data expressed as percent change from littermate euploid mice. Box whisker plots show median (solid lines), means (dashed lines), 90% confidence limits (box) and range (whiskers), n=5-6 preparations, 2-4 brains/preparation. Redline marks 0% change.

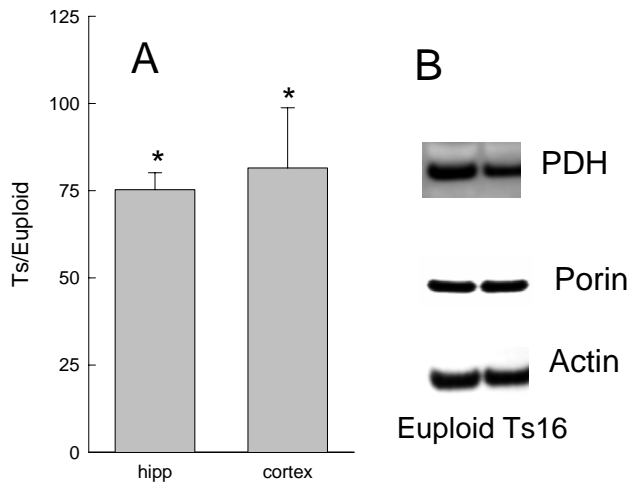


Figure 5. A: Ts16 PDH immunoreactivity in hippocampus and cortex homogenates normalized to porin and expressed as a percent of euploid, means and SD for 4 experiments. * Ts16 is significantly different from euploid for the raw PDH/porin ratios by paired t-test ($p < 0.05$). B. Representative Western blot results for one experiment using cortical homogenates.

The pyruvate dehydrogenase (PDH) complex in the inner mitochondrial matrix has been shown to be a target of oxidative stress. The PDH complex consists of 3 major protein subunits. Expression of the E1 α subunit has been shown to be reduced by oxidative stress following cardiac arrest-resuscitation (Martin et al., 2005). Western blots for PDH in euploid and Ts16 cortex and hippocampus (Figure 5) showed a significant reduction in PDH in Ts16 in both brain regions. Taken together with the results for ETC subunit expression, this shows a pattern of changes that is most consistent with an increased oxidative stress in Ts16 and not with a signal transduction error that affects a specific protein. A manuscript on these experiments will be submitted by November, 2006.

NAD(P)H fluorescence in Ts16 hippocampal neurons.

Pyridine nucleotide (NADH and NADPH) fluorescence can be used to measure metabolism on a cell-by-cell basis using fluorescence microscopy because the reduced form

NAD(P)H is fluorescent while the oxidized form, NAD(P) is not (Shuttleworth et al., 2003). Experimentally, euploid and Ts16 hippocampal neurons were grown on coverslips and imaged in a superfusion chamber (Figure 6A). When KCN is applied the ETC can no longer give electrons to oxygen and NAD(P)H usage for respiration stops, shown as a rise in the fluorescence (Figure 6B,C). When the protonionophore FCCP is applied, the ETC runs at its maximal rate and all the mitochondrial NAD(P)H is oxidized to NAD(P) and the fluorescence falls. This delta between the KCN maximum and the FCCP minimum signal is a measure of the

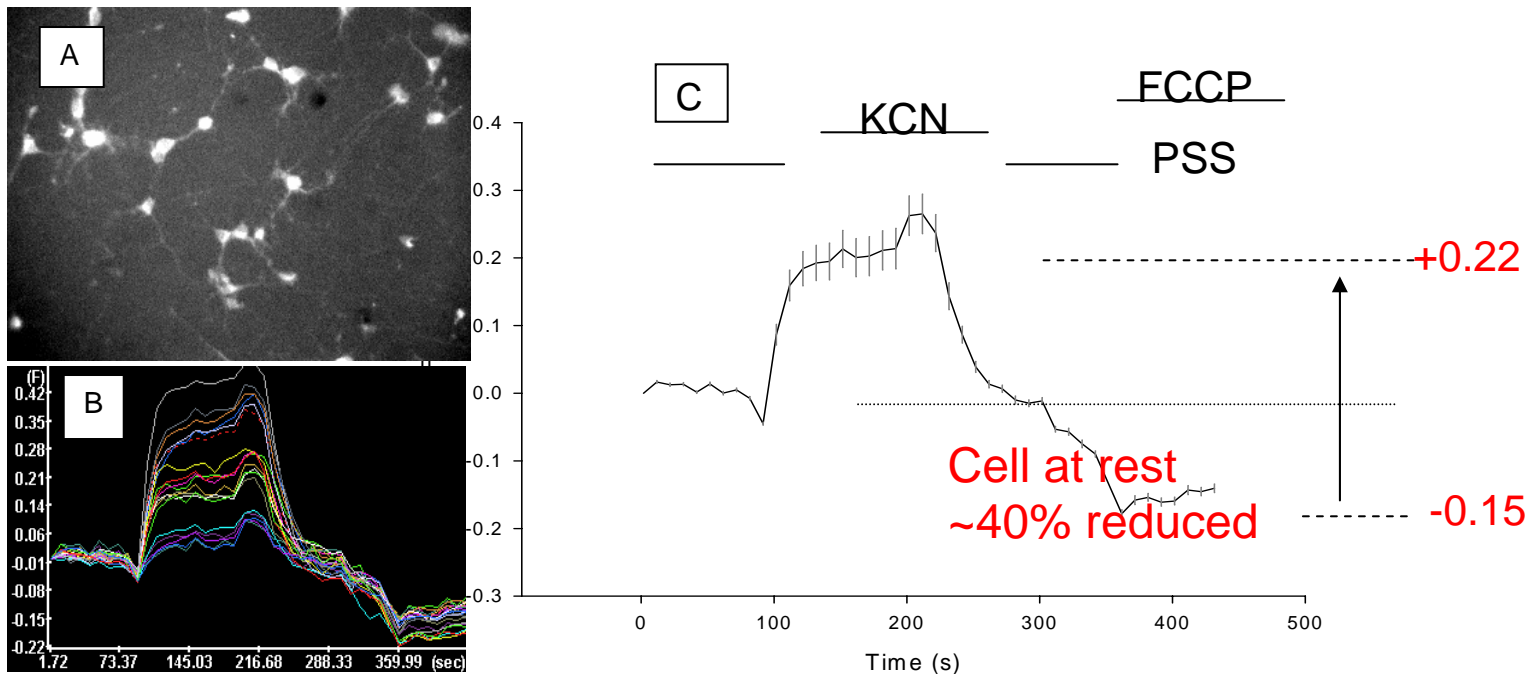


Figure 6: A: NAD(P)H fluorescence in a field of hippocampal neurons. B: changes in fluorescence as dF/F_o (F_o is fluorescence at start of experiment, F and F_o are fluorescence after background subtraction) during the experiment in C. C. mean and SEM of the cells in A and B during FCCP and KCN administration. The maximum delta in this experiment is 0.37 ($0.22 + 0.15$).

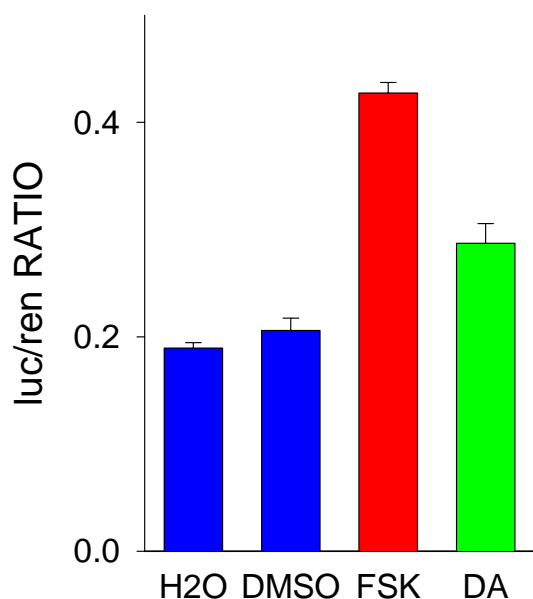


Figure 7: Stimulation of BDNF-luciferase reporter gene expression in astrocytes by 50mM forskolin (FSK, 2.3-fold) and 0.15mM dopamine (1.4-fold). Data are luciferase counts normalized to renillin expressed as means and SEM for triplicates in one representative experiment, similar results were seen in three independent experiments.

mitochondria NAD(P)H in the cell. Using this assay, I have found decreased pyridine nucleotides in Ts16 hippocampal neurons suggesting decreased production or increased consumption of the compounds in Ts16. Interestingly, increased consumption of pyridine nucleotides by PARP is a feature of oxidative stress. Decreased pyridine nucleotides in Ts16 would predispose the neurons to further oxidative damage since NADH is required for reduction of glutathione, a major cellular antioxidant. This finding opens up the possibility of a new neuroprotective therapy using PARP inhibitors to rescue Ts16 neurons. Experiments with the PARP inhibitor DPQ are currently in progress.

BDNF expression in astrocytes in Parkinson's disease.

The following data are included here because, although not a part of the initial grant, these experiments were begun due to the current study's focus on PD and the trkB/BDNF system. Further experiments (not funded by this grant) are in progress and, if successful, a new grant on this topic will be submitted.

It is known that astrocytes can synthesize BDNF and that BDNF synthesis can be induced by dopamine (see e.g., Ohta et al., 2004). BDNF gene expression is complicated with 7 mRNAs generated from one gene (Liu et al., 2006). These differ in their upstream noncoding exons and contain different promoter and regulatory sequences that are thought to be important in directing responses to different stimuli and, possibly, in directing transport of the mRNAs to different sites, e.g. the synapse, for translation. Nothing is known about which mRNAs are involved in dopamine-mediated BDNF synthesis in astrocytes, although

preliminary evidence in our group suggests that there are significant differences between astrocytes and neurons. My interest in BDNF and trkB has led me to the following hypothesis: *in PD when dopamine levels drop, this leads to decreased BDNF expression by striatal and substantia nigra astrocytes and this decreased BDNF contributes to neuron loss and dysfunction in PD.* Increasing astrocyte BDNF expression may be protective in PD and may be a more viable approach than increasing neuron BDNF since the neurons are being themselves damaged, which may reduce their ability to synthesize new BDNF.

The following experiments demonstrate some of the tools needed for the first phase of this study. Using a luciferase-BDNF reporter construct (see methods in Kingsbury et al., 2003), both forskolin and dopamine stimulation of BDNF expression can be detected (Figure 7). This finding may underestimate the amount of BDNF synthesized since this reporter was made using the upstream sequence from just one of the 7 possible combinations. New reporters for the other BDNF mRNAs are being generated. Real time PCR probes can be made for the individual isoforms. A preliminary pair of probes targeting the coding exon common to all the mRNAs has been successfully made and used to detect BDNF in our cultured astrocytes. Future experiments will determine 1. BDNF mRNA expression in astrocytes in control and dopamine treated cells; 2. Using pharmacology, which dopamine receptor isoforms are involved and is there any correlation between activation of specific dopamine receptors and BDNF isoform choice? 3. Using siRNA to block astrocyte BDNF synthesis, does this reduce astrocyte-mediated neuron protection in vitro?

Key Accomplishments

- In Ts16 brain there are decreased levels of a labile subunit of Complex I of the electron transport chain (ETC) and a pattern of lower levels of expression of other ETC proteins in Ts16 mitochondria that is not due to lower numbers of mitochondria in Ts16.
- In Ts16 there is also a decrease in expression of the oxidative stress-sensitive matrix protein subunit E1a of pyruvate dehydrogenase (PDH).
- At the cellular level, individual hippocampal neurons have lower levels of pyridine nucleotides, reflecting increase oxidative stress and a susceptibility to further stress in Ts16.
- In this year I also published two papers (Bambrick et al 2006,a; Dorsey, 2006) with two more to be submitted by the end of 2006 (Bambrick et al, 2006b; Kingsbury et al., 2006).

Conclusions

The pattern of decreases in many mitochondrial proteins without a change in porin suggests that in Ts16 the number of mitochondria is similar to that in euploid brain but that ongoing oxidative stress has led to damage and increase degradation of susceptible subunits, especially in Complex I and PDH—both of which are known to be sensitive to oxidative stress. Interestingly, cytochrome oxidase—Complex IV—is less sensitive to this stress. The lower pyridine nucleotide levels in Ts16 neurons may also reflect increased oxidative stress; whether protecting pyridine nucleotides levels can rescue Ts16 neurons from apoptosis will be tested using PARP inhibitors.

References

- Bambrick, L.L. and Krueger, B.K. (1999) Neuronal apoptosis in mouse trisomy 16:mediation by caspases. *Journal of Neurochemistry* 72:1769-1772.
- Bambrick LL, Krueger BK, Fiskum G (2005) Mitochondrial function in the trisomy 16 mouse cortex. *Society for Neurosciences Abstracts* 30: 1013.12.
- Bambrick L.L, Mehrayban Z, Chandrasekaran K, Wright C and Fiskum G. (2006a) Calcium-induced mitochondrial dysfunction in cortical astrocytes and cerebellar granule neurons *Journal of Bioenergetics and Biomembranes* 38: 42-47.
- Bambrick LL, Krueger BK, Fiskum G (2006b) Oxidative stress causes protein damage and impaired Complex I-mediated respiration in mouse trisomy 16 cortex (in preparation for *J Neurochem*).
- Dorsey SG, Bambrick LL, Balice-Gordon RJ, Krueger BK. (2002) Failure of brain-derived neurotrophic factor-dependent neuron survival in mouse trisomy 16. *J.Neurosci.* 22:2571-2578.
- Dorsey SG, Renn CL, Carim-Todd L, Barrick CA, Bambrick L, Krueger BK, Ward CW, Tessarollo L. (2006) In vivo restoration of physiological levels of truncated TrkB.T1 receptor rescues neuronal cell death in a trisomic mouse model. *Neuron* 51: 21-8.
- Kingsbury TJ, Murray PD, Bambrick LL, Krueger BK. (2003) Ca(2+)-dependent regulation of TrkB expression in neurons. *Journal of Biological Chemistry* 278: 40744-8.
- Kingsbury TJ, Bambrick L, Krueger BK. Calcineurin Activity allows stimulation of CREB transcription in neurons.
- Liu QR, Lu L, Zhu XG, Gong JP, Shaham Y, Uhl GR. (2006) Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine. *Brain Res* 1067: 1-12.
- Martin E, Rosenthal RE, Fiskum G. (2005) Pyruvate dehydrogenase complex: metabolic link to ischemic brain injury and target of oxidative stress. *J Neurosci Res* 79: 240-7.
- Ohta K, Kuno S, Mizuta I, Fujinami A, Matsui H, Ohta M. (2003) Effects of dopamine agonists bromocriptine, pergolide, cabergoline, and SKF-38393 on GDNF, NGF, and BDNF synthesis in cultured mouse astrocytes. *Life Sci* 73: 617-26.
- Schuchmann S, Heinemann U (2000) Increased mitochondrial superoxide generation in neurons from trisomy 16 mice: A model of Down's syndrome. *Free Radical Biol.Med.* 28: 235-250.
- Shuttleworth CW, Brennan AM, Connor JA. (2003) NAD(P)H fluorescence imaging of postsynaptic neuronal activation in murine hippocampal slices. *J Neurosci* 23: 3196-208.